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Molecular Epidemiology of Mastitis Pathogens of Dairy Cattle and Comparative Relevance to Humans

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Abstract Mastitis, inflammation of the mammary gland, can be caused by a wide range of organisms, including gram-negative and gram-positive bacteria, mycoplasmas and algae. Many microbial species that are common causes of bovine mastitis, such as *Escherichia coli*, *Klebsiella pneumoniae, Streptococcus agalactiae* and *Staphylococcus aureus* also occur as commensals or pathogens of humans whereas other causative species, such as *Streptococcus uberis*, *Streptococcus dysgalactiae* subsp. *dysgalactiae* or *Staphylococcus chromogenes*, are almost exclusively found in animals. A wide range of molecular typing methods have been used in the past two decades to investigate the epidemiology of bovine mastitis at the subspecies level. These include comparative typing methods that are based on electrophoretic banding patterns, library typing methods that are based on the sequence of selected genes, virulence gene arrays and whole genome sequencing projects. The strain distribution of mastitis pathogens has been investigated within individual animals and across animals, herds, countries and host species, with consideration of the mammary gland, other animal or human body sites, and environmental sources. Molecular epidemiological studies have contributed considerably to our understanding of sources, transmission routes, and prognosis for many bovine mastitis pathogens and to our understanding of mechanisms of host-adaptation and disease causation. In this review, we summarize knowledge gleaned from two decades of molecular epidemiological studies of mastitis pathogens in dairy cattle and discuss aspects of comparative relevance to human medicine.

Keywords Molecular epidemiology · Bovine · Mastitis · Strain typing · Outbreak investigation · Genome · Host adaptation

Abbreviations

PCR	polymerase chain reaction
CoNS	coagulase negative staphylococci
MLST	multi-locus sequence typing
RAPD	random amplified polymorphic DNA
ERIC	enterobacterial repetitive intergenic consensus
PFGE	pulsed-field gel electrophoresis

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rep-PCR	repetitive DNA sequence PCR
GBS	group B streptococcus
ST	sequence type
SLV	single locus variant
CC	clonal complex
LGT	lateral gene transfer
MLVA	multiple loci VNTR analysis
VNTR	variable number of tandem repeats
MGE	mobile genetic element
SEZ	*Streptococcus equi* subsp. *zooepidemicus*
RFLP	restriction fragment length polymorphism
SE	staphylococcal enterotoxin
TSST	toxic shock syndrome toxin
AFLP	amplified fragment length polymorphism
RMA	resolution melting analysis
REA	restriction enzyme analysis

Introduction

Over the past two decades, a wide range of phenotyping and genotyping methods have been developed or implemented to study mastitis-causing bacteria of dairy cattle at the species and subspecies level. Genotyping methods used to characterize bovine mastitis-causing pathogens range from simple restriction digest or PCR based approaches to micro-arrays and whole genome sequencing. It is beyond the scope of this paper to explain technical aspects, strengths and weaknesses of all molecular methods used for typing of bovine mastitis pathogens. The reader is referred to guidelines for the validation and application of typing methods for use in bacterial epidemiology for technical aspects [180, 189, 194] and to a review of use of molecular epidemiology in veterinary practice for applications and interpretations [212]. The focus of our paper is "molecular epidemiology", which we interpret as the use of DNA-based characterization of micro-organisms at the subspecies level to understand their sources, transmission routes, biological relationships, and virulence characteristics. Molecular diagnostics at the species level will not be covered in depth, unless the state of the art for a particular species has not progressed much beyond that stage. Molecular determinants of antimicrobial resistance are beyond the scope of this review.

The main bovine mastitis pathogens that have been investigated using molecular methods are the gram-negative species *Escherichia coli* and *Klebsiella pneumoniae* and the gram-positive species *Streptococcus agalactiae*, *Streptococcus uberis*, and *Staphylococcus aureus*. Most of these organisms also occur as commensals or pathogens of humans. Development of molecular methods for use in human medicine has facilitated studies of these organisms in the context of bovine mastitis. For other species or genera, such as *Streptococcus dysgalactiae* subsp. *dysgalactiae*, coagulase negative staphylococci (CoNS) or *Mycoplasma* spp., molecular typing at the subspecies level is still in its infancy. At the forefront of molecular epidemiological research, large scale studies using library typing methods and on-line databases such as multilocus sequence typing (MLST) demonstrate host-adaptation of major pathogens of cattle and humans, whilst virulence gene arrays and whole genome sequencing shed light on mechanisms of pathogen evolution and adaptation to the bovine host or the mammary gland. Meanwhile, comparative typing methods based on electrophoretic banding patterns are increasingly used in veterinary diagnostic laboratories, bringing the use of molecular epidemiology for outbreak- and farm-investigations within reach of dairy veterinarians and farm advisors.

The aim of this review is to summarize knowledge gleaned from two decades of molecular epidemiological studies of mastitis pathogens in dairy cattle, reflecting the breadth of coverage and depth of knowledge available for the different pathogen species. For each species or genus, major research questions, results and insights will be summarized, starting with gram-negative pathogens, followed by gram-positive pathogens, and ending with algae and mycoplasmas. Animal- and herd-level studies as well as studies spanning a wide spatiotemporal scale or multiple host species are considered. Where relevant, comparative aspects of human medicine will be discussed.

E. coli Mastitis

Escherichia coli is a common cause of intramammary infection in dairy cattle. Infection usually manifests with clinical signs. Based on epidemiological data and early strain typing studies, which showed large heterogeneity among isolates associated with cases of mastitis within farms, [103, 109, 133], *E. coli* is classified as an opportunistic environmental pathogen. There are no specific virulence factors that differentiate strains with the ability to cause mastitis from other *E. coli* strains [12, 183, 206]. The severity of clinical signs, which may range from mild to fatal, is largely attributed to host-characteristics [29].

Most cases of *E. coli* mastitis are transient and end with death of either the host or the pathogen. However, recurrent cases of clinical *E. coli* mastitis were recognized in early strain typing studies [103, 109]. Recurrent cases could be due to repeated episodes of infection and cure, or to persistent infection with alternating subclinical and clinical episodes. Considering the heterogeneity of environmental *E. coli*, repeated episodes of infection would be expected to be caused by different strains. Such repeated episodes could be due to chance or to increased host-level or quarter-level

susceptibility to infection. To be considered persistent, an intramammary infection would have to be caused by a single strain that was present for a long time, resulting in repeated isolation of the same strain from multiple clinical episodes. In an initial study of 7 herds, persistent *E. coli* infections, characterized by repeated isolation of the same random amplified polymorphic DNA (RAPD) type from multiple clinical episodes, were described as "sporadic" [103, 109]. In a subsequent study of 300 herds, 11% of cows had recurrent clinical cases of *E. coli* mastitis [43]. Half of the recurrent cases occurred in the same mammary quarter as the initial case, and half of the recurrent cases within a mammary quarter were due to the same strain as the initial case. This shows that both scenarios, i.e. repeated infections and persistent infection, do indeed occur. When recurrent cases occurred in a different quarter than the initial episode, strains were still the same for approximately 28% of quarters, suggesting that within-cow transmission of *E. coli* may occur [43]. Whether apparent transmission occurs via direct or indirect contact between teats or results from concurrent exposure is unknown. Systemic dissemination is unlikely, because cows with systemic *E. coli* infection tend to be very sick, whereas cows with recurrent clinical episodes generally show relatively mild symptoms [206]. In 6 dairy herds in England, Bradley and Green [22] observed the same phenomena as described by Döpfer and colleagues [43], albeit at different frequencies. In their study, the same strain of *E. coli* accounted for 86% of recurrent cases within a quarter, but only 8.5% of recurrent cases in different quarters of the same animal [22]. In both studies, strain typing was based on use of enterobacterial repetitive intergenic consensus (ERIC) sequence primers [22, 43]. Therefore, differences in results are likely to be due to herd selection rather than typing methods.

The high incidence of clinical *E. coli* mastitis in early lactation has been attributed to increased host susceptibility at that time [29]. Using ERIC-typing, however, many clinical episodes of *E. coli* mastitis in early lactation could be traced back to infections that originated in the preceding non-lactating period rather than the lactating period [21]. This discovery led to evaluation of antimicrobial products with a gram-negative spectrum for treatment and prevention of mastitis during the non-lactating period. Use of such a product reduced the incidence of clinical *E. coli* mastitis during the non-lactating period as well as the first 100 days of the following lactation [23]. This is a nice example of molecular studies leading to increased understanding of the epidemiology and pathogenesis of mastitis or even "shattering a paradigm", and subsequent development of interventions targeting the newly understood or rediscovered biological mechanisms. Although it has been suggested that the occurrence of persistent *E. coli* infections may be due to host-adaptation of the pathogen [22], persistent *E. coli* infections with recurrent clinical signs were already described in the 1970s [84]. Molecular typing methods were not available at the time, but serology was used to demonstrate that repeated clinical episodes were due to the same serotype of *E. coli*. Whether the incidence of persistent infections with recurrent clinical episodes has increased, or whether our awareness has increased is difficult to ascertain with available data.

Despite clear differences in phenotypic traits in vitro, particularly with regards to invasion and survival in mammary epithelial cells [3, 44], no clear genetic differences have been identified between *E. coli* strains from transient or persistent infections [42, 183]. Phenotypic differences have also been described between *E. coli* isolates from cows with clinical mastitis and the farm environment of those cows [18]. The two groups of isolates differed in average lactose fermentation and growth in milk (both were higher in mastitis isolates) and in phagocytosis by bovine polymorphonuclear neutrophils, which are considered to be the first line of defense against coliform mastitis (lower for mastitis isolates; [18]). In addition to phenotypic differences, clustering of pulsed-field gel electrophoresis (PFGE) types within categories (mastitis or environmental) was described [18]. It is difficult to infer phylogeny from PFGE data, or to reconcile the data from Blum and coworkers with those from other studies. So far, all methods used for *E. coli* typing have targeted pre-selected elements of the genome only, e.g. virulence or antimicrobial resistance genes, restriction sites, or primer binding sites. Whole genome sequencing of transient and persistent *E. coli* strains is in progress and may shed new light on possible mechanisms of host adaptation in mastitis-causing *E. coli*.

Klebsiella Mastitis

The most common *Klebsiella* species causing bovine mastitis are *K. pneumoniae* and *K. oxytoca*. Molecular methods, specifically sequencing of the *rpo*B gene, recently showed that some isolates with the phenotypic appearance of *K. pneumoniae* or *K. oxytoca* belong to the closely related genus *Raoultella*. Both *R. terrigena* and *R. planticola* can be found in the dairy farm environment, and *R. planticola* has also been isolated from milk [129, 220]. Most molecular epidemiological studies of *Klebsiella* focus on assessment of heterogeneity of *K. pneumoniae* within samples or herds, or on comparison of isolates obtained from animals and their environment to identify *Klebsiella* sources and transmission routes.

Analysis of *Klebsiella* populations within individual milk samples has yielded conflicting results. Paulin-Curlee and colleagues [141] characterized 3 isolates for each of 26 milk samples and found a single repetitive DNA sequence

PCR (rep-PCR) type in approximately 47% of samples, 2 rep-PCR types in 36% of samples and 3 rep-PCR types in 18% of samples. By contrast, Munoz and coworkers [129] characterized 4 isolates for each of 14 milk samples and consistently found a single RAPD-type per sample. Milk samples in the first study originated from a privately-owned dairy herd and were mailed to a diagnostic laboratory. Details on sample collection procedures are not given. Milk samples in the second study were collected by trained field personnel from a different diagnostic laboratory, using aseptic technique. The number of strains per sample may differ between herds if they are sampled by different people [142]. Given that *Klebsiella* is very common on teat skin of dairy cattle [130], sample collection methods may play a role in heterogeneity of *Klebsiella* in milk samples. The observed strain heterogeneity may also be affected by the discriminatory power of the typing method, which differs between methods, or even between primer sets used for a single method, such as RAPD-typing [128]. It has been suggested that the discriminatory power of rep-PCR, PFGE and MLST increases in that order for *K. pneumoniae*, but values for Simpson's index of discrimination, a standard measure of discriminatory power [180], were not based on analysis of the same collection of isolates with each method but rather on nested subsets of isolates [142]. This may have affected the apparent discriminatory power of the methods.

In samples from bovine feces, the rumen, drinking water and the farm environment, within-sample heterogeneity is common [129]. Based on characterization of 4 isolates per fecal sample using RAPD-typing, the median number of strains per sample was estimated at 3 [128]. With characterization of 5 isolates per fecal or rumen sample and using the same method, the median number of strains per sample was estimated at 4 [220]. This illustrates that the number of strains found is a function of the number of isolates processed, which tends to be a function of the budget for a particular study. If it is assumed that a fecal sample contains 4 strains in equal numbers, as many as 15 isolates need to be characterized to be 95% certain that all strains will be detected [45]. Considering the heterogeneity of strains in cows, their feces and the farm environment, it can be difficult to determine the origin of a mastitis-causing strain or, conversely, the mastitis-causing potential of strains from extra mammary sources. Wood-based bedding is often cited as a source of *Klebsiella* and has been implicated in mastitis outbreaks based on culture results [134, 167]. In a study of 6 Belgian herds, Verbist and colleagues [199] characterized isolates at the sub-species level to assess whether *Klebsiella* from feces or sawdust could be identified as the cause of mastitis. Among 120 fecal *K. pneumoniae* isolates, 88 PFGE patterns were identified. None of these patterns were associated with clinical mastitis during the study. In unused bedding material, 5 isolates were detected, each with a unique PFGE pattern. Again, none of these patterns were associated with clinical mastitis during the study. In another attempt to match *Klebsiella* from sawdust with mastitis cases, only *Raoultella* was found in unused bedding [129]. Used bedding material, however, has been identified as a possible source of infection. It is thought that presence of *Klebsiella* in used bedding is due to contamination with bovine feces or with milk from *Klebsiella* infected cows [129, 199].

As for *E. coli*, the heterogeneity of *Klebsiella* strains in the environment is reflected by strain heterogeneity among infected cows within a herd [99, 129, 141, 142]. Even so, strains affecting multiple cows have been observed in several herds [99, 129, 142]. Possible explanations include lack of discriminatory power of typing methods, cow-to-cow transmission, exposure to a point source, or increased fitness of specific strains [212]. Lack of discriminatory power is difficult to exclude, although multiple methods were used in the studies cited (plasmid profiling, rep-PCR and RAPD-typing) and each method identified within-farm heterogeneity. Attempts to demonstrate cow-to-cow transmission via the milking machine, which is the usual mode of transmission for mastitis pathogens, appeared promising based on culture results. Molecular typing, however, showed that apparent transmission was caused by contamination of the milking machine with different strains of *Klebsiella* by different cows, possibly from teat skin [129, 130]. Excretion of milk by an infected cow, resulting in seeding of bedding with a large number of colonies from a single strain, was considered the most likely explanation for the observations and was tentatively called "cow-to-cow transmission via the environment" [129]. To date, markers of increased fitness, i.e. enhanced ability to cause mastitis, have not been published for *Klebsiella* strains.

Other Gram-Negative Pathogens

Non-coliform gram-negative species may occasionally cause severe mastitis problems. *Pseudomonas aeruginosa* has been associated with mono- or polymicrobial breast abscesses and septic mastitis in women [74, 126] and with mastitis in cattle, sheep and goats [173]. Outbreaks of *P. aeruginosa* mastitis in dairy cattle have been reported from Australia [119], Ireland [34], Israel [173] and The Netherlands [177], often with a high fatality rate. Based on PFGE, a large variety of *P. aeruginosa* strains may cause mastitis in sheep, goats and cattle, without evidence of clonality within herds [173]. In one case, clonality was suggested based on exposure to a common risk factor: all herds involved in the Dutch multi-herd *P. aeruginosa* mastitis outbreak used the same disinfectant wipes [177]. Such

wipes are used to disinfect teat ends before application of intramammary antimicrobial treatment via the teat opening. Strain typing data were not generated to test the hypothesis of clonality of this outbreak. A similar multi-herd outbreak, associated with the same brand of disinfectant wipes, occurred in Ireland [34]. Molecular epidemiological investigations demonstrated that all herds were affected by the same strain of *P. aeruginosa*. Initially, this result was obtained using ribotyping with the restriction enzyme *Cla*I. Comparison with epidemiologically unrelated human isolates from hospitals demonstrated that the same *Cla*I ribotype could also be found in humans, suggesting that lack of discriminatory power might explain the observed strain homogeneity. Subsequent ribotyping with *Pvu*II proved more discriminatory, with only the mastitis isolates showing identical patterns. The outbreak strain was also isolated from an unused container of the suspect disinfectant wipes, confirming that they were the likely source of the outbreak. Without molecular typing, identification of a common risk factor should not be interpreted as evidence of that risk factor being the source of the outbreak. This was demonstrated in a multi-state outbreak of *Serratia* mastitis [127]. In this outbreak, as in the Dutch *P. aeruginosa* outbreak, a common risk factor was identified across herds, i.e. use of a chlorhexidine based teat dip. In contrast with the *P. aeruginosa* outbreak, isolates from the *Serratia* outbreak did not belong to a single strain type. Based on RAPD-typing, *Serratia* isolates from different farms belonged to different strains, showing that the suspect product had been contaminated on the individual farms. Within each farm, animals were usually infected with a single strain of *S. marcescens* (Fig. 1) and the same strain was found in teat dip on some farms. This shows that the teat dip may have acted as a point source or a fomite for transmission. In other herds, multiple *S. marcescens* strains

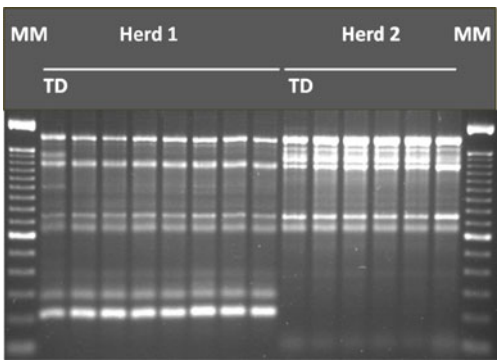

Figure 1 Random amplified polymorphic DNA profiles of *Serratia marcescens* isolates from two dairy herds (indicated by *lines*). Lanes 1 and 16 contain molecular markers (MM); Lanes 2 and 10 represent teat dip isolates (TD); Lanes 3 through 9 and 11 through 15 represent isolates from composite cow milk samples. Within-herd homogeneity and between-herd heterogeneity are seen

or multiple *Serratia* species were identified, demonstrating that *Serratia* mastitis can also result from exposure to a variety of environmental strains of the pathogen. In hospitals, as on farms, outbreaks of *Serratia* infection are occasionally linked to contaminated chlorhexidine based on bacteriology and molecular typing [115, 200]. *Serratia* mastitis is very rare in humans, but a case was recently described in association with a contaminated breast pump [54].

Streptococcus agalactiae

In humans, *Strep. agalactiae* or group B streptococcus (GBS) is associated with early and late onset disease in infants, with asymptomatic colonization of the urogenital and gastro-intestinal tract, and with septicemia and other clinical manifestations in non-pregnant adults [94, 105]. In women, *Strep. agalactiae* may cause breast abscess formation and clinical or asymptomatic mastitis [159]. Maternal mastitis is a risk factor for late onset *Strep. agalactiae* disease in infants [38, 138, 201]. When molecular typing of *Strep. agalactiae* from mother-baby pairs is performed, isolates within pairs are indistinguishable but distinct from those of other pairs, confirming mother-child relationships [15, 37, 100]. In cattle, mastitis is the only disease associated with *Strep. agalactiae* infection. Transmission within herds is thought to be strictly contagious, i.e. from cow to cow, due to insufficient hygiene in the milking parlor, allowing multiple animals to come into contact with equipment, hands or towels that are contaminated by milk from an infected cow ([97, 132]). This mode of transmission results in the presence of a single strain in multiple animals in a herd [11, 37, 47, 120, 136, 179, 203]. The observed strain homogeneity is not due to lack of discriminatory power, because the same techniques have demonstrated differences between isolates from different farms or host species, including humans, cattle and fish [11, 47, 143]. Because herd-level mastitis problems are generally easily and cost-effectively resolved through implementation of herd hygiene and treatment programs [49, 52], few molecular epidemiological studies of persistence, transmission routes or sources of infection have been conducted in cattle.

The major question in molecular epidemiology of bovine *Strep. agalactiae* is whether it constitutes a human health hazard, either through direct transmission between cattle and humans [26, 112] or through evolution of human-pathogenic strains from a bovine reservoir [16]. Conversely, others have suggested that humans may act as a source of infection for cattle [41, 212], a mechanism that has also been proposed for dogs, cats and crocodiles [17, 210]. Experiments to assess the pathogenic potential of human *Strep. agalactiae* in cattle were already conducted in the

early 1980s, before molecular typing was feasible. Challenge of lactating quarters with human *Strep. agalactiae* resulted in clinical mastitis, but human strains showed a pronounced tendency to spontaneously clear [92, 196]. In contrast, bovine strains caused chronic subclinical infection, enhancing the probability of subsequent spread within the herd [92]. The first large-scale molecular comparison of human and bovine *Strep. agalactiae* populations was based on RAPD-typing. The majority of bovine isolates were contained in major RAPD clusters that consisted exclusively of bovine isolates but some clusters included a mixture of bovine and human isolates [113]. Subsequent comparisons using other methods, such as ribotyping, PFGE and molecular serotyping supported the notion that human and bovine *Strep. agalactiae* are largely distinct populations [41, 48, 181, 222]. The distinction is not always absolute. For example, virulence genes such as the C5a peptidase gene *spc*B and the laminin binding gene *lmb* are present in almost all human isolates and in 20 to 44% of bovine isolates [27, 64, 179, 210].

When MLST was developed for *Strep. agalactiae*, strain typing information could be used to investigate population biology and pathogen evolution [93]. MLST based grouping did not correspond to grouping of strains based on RAPD or serotyping [19, 20, 27], but an association between sequence types (STs) and host species or clinical manifestation was identified [16, 53, 93]. The major human STs (ST1, ST17, ST19 and ST23) were initially associated with asymptomatic carriage, invasive neonatal disease, asymptomatic carriage, and a mixture of invasion and carriage, respectively [93]. Subsequent studies only supported the association of ST17 with neonatal invasive disease [20, 94]. Human isolates showed greater diversity in STs than bovine isolates and clustered separately from bovine isolates, which were primarily comprised of ST67 and its single locus variant (SLV) ST61 [16]. Given that many organ systems can be affected by *Strep. agalactiae* in humans while only the mammary gland is affected in cattle, it may not seem surprising that a lower level of genetic heterogeneity was detected in bovine isolates. It was suggested that the hyperinvasive human neonatal clone ST17 had arisen from bovine ST67, to which it is connected by a chain of 2 SLVs [16]. Despite the relatively close relationship between ST17 and ST67, use of additional markers such as insertion sequences and *inf*B alleles still identified human and bovine isolates as largely distinct populations [19]. Subsequent studies considered a much larger part of the genome, either through combination of an expanded 15-gene MLST scheme, molecular serotyping and virulence gene screening [179] or by comparative genomic hybridization [27]. Both studies showed that the *Strep. agalactiae* genome has a composite structure due to recombination, which distorts the phylogenetic signal. These studies did not support the idea that ST17 had arisen from a bovine ancestor and reaffirmed that human and bovine isolates largely form distinct populations [27, 179]. In addition to isolates from clonal complex (CC) 67, isolates from CC23 are frequently found in bovine mastitis [19, 27, 73, 179]. ST23 probably has the widest host range of all *Strep. agalactiae* STs. It has been found in humans, cattle, dogs, crocodiles and grey seals [17, 27]. Within ST23, however, human and bovine specific subpopulations can be distinguished based on serotyping and the diversity of *inf*B, *sod*A and *gdh* alleles [179]. Other strains that are primarily associated with humans but have been reported from cattle include members of CC1, ST8, CC19 and CC26 [27, 73, 112, 136, 179].

Earlier this year, the first complete genome sequence of a bovine *Strep. agalactiae* isolate, belonging to ST67, was published [162]. Comparison with genomes from human *Strep. agalactiae* isolates revealed 8 novel genomic islands that were probably acquired by lateral gene transfer (LGT). Screening of 20 bovine and 20 human isolates showed that some of these islands were significantly more common in bovine than in human isolates. One such island includes genes from a fructose- and lactose-operon, in agreement with studies from the pre-molecular era, which described differences between human and bovine isolates in lactose utilization [56, 162]. Using 238 isolates from 9 countries and 5 continents, Sørensen and colleagues [179] demonstrated that 92% of bovine isolates and 13% of human isolates ferment lactose. The linked fructose- and lactose-operons of *Strep. agalactiae* share 99% sequence homology with those of *Strep. dysgalactiae* subsp. *dysgalactiae*, another bovine mastitis pathogen. This suggests that LGT may take place between different pathogen species in the bovine udder. Similarly, LGT between *Strep. agalactiae*, *Strep. pyogenes* and group C and G streptococci is thought to have contributed to adaptation to their shared human niche [64]. Another example of LGT between bovine mastitis pathogens is provided by the nisin-operon, which shows close similarity between *Strep. agalactiae* and *Strep. uberis*, a pathogen that is rarely associated with any other disease or host species [162, 211].

Evolutionary considerations regarding niche adaptation are important for epidemiological reasons. If bovine *Strep. agalactiae* forms a reservoir for emergence of virulent human clones, an argument could be made for eradication of bovine *Strep. agalactiae* [86]. If occurrence of *Strep. agalactiae* in predominantly *Strep. agalactiae*-negative herds or areas is due to spill-over of human *Strep. agalactiae* into the bovine population [41], eradication of bovine *Strep. agalactiae* may not be possible or necessary. Pathogen evolution may also help to explain the re-emergence of *Strep. agalactiae*, a phenomenon observed in Denmark. In the 1950s, Denmark had a herd-level prevalence of *Strep. agalactiae* of 20 to 30%. Due to

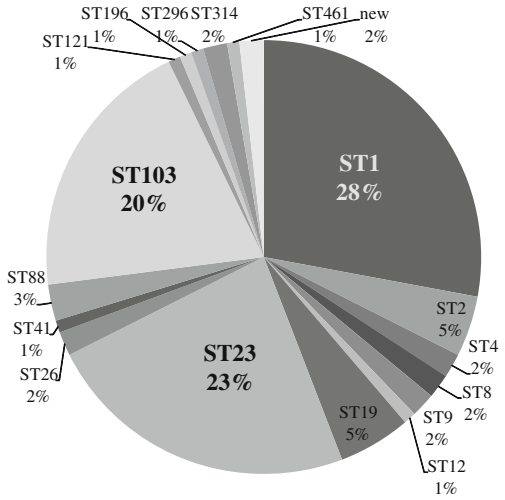

Figure 2 Frequency distribution of sequence types (ST) of *Streptococcus agalactiae* isolates from bulk tank milk originating from 111 dairy farms in Denmark, 2009, showing predominance of ST1, ST23 and ST103

systematic control efforts, prevalence had decreased to approximately 2% by 1979 [91] and it stayed at that level through the 1980s and '90s [4]. Since 2000, a steady increase in prevalence of *Strep. agalactiae* has occurred and in 2008 close to 6% of herds were positive [96]. MLST of 111 isolates from a bulk tank survey conducted in Denmark in 2009 showed that the most common strains were ST1 (28%) and ST23 (23%), which are STs that were previously primarily associated with human infection. Members of CC67 were not detected (RNZ and JK, unpublished; Fig. 2). The third most common ST was ST103, which has occasionally been isolated from humans, a cat, a guinea pig and dairy cattle [20, 27, 73]. It is unknown why or how ST103 emerged as a highly prevalent clone in bovine milk. Acquisition of genetic material that confers a survival advantage in the bovine udder is one of the explanations under investigation.

Streptococcus uberis

Streptococcus uberis is strictly an animal pathogen [211] and fewer typing methods are available than for *Strep. agalactiae* or *Staph. aureus*, which are also human pathogens. Early studies of *Strep. uberis* used comparative methods such as RAPD-typing, rep-PCR or PFGE. Later, 2 MLST schemes were developed. The first MLST scheme encompassed a mixture of housekeeping genes, virulence genes and vaccine targets [218]. The second MLST scheme was based on housekeeping genes only [33], although the status of one of the genes, *yqi*L, as housekeeping gene has been cast in doubt due to its absence from some *Strep. uberis* strains [193]. An MLVA scheme (Multiple Loci VNTR Analysis; VNTR, Variable Number of Tandem Repeats; [66]) has also developed but, like the first MLST scheme, it has not been applied widely. The genome sequence of *Strep. uberis* O140J, a strain often used for challenge studies [9, 55, 204], was made publicly available in 2009 [205]. This was quickly followed by whole genome comparisons of multiple strains using a DNA-microarray with *Strep. uberis* O140J as reference strain [104]. The majority of molecular epidemiological studies on *Strep. uberis* focus on sample-, cow- and herd-level strain heterogeneity with the aim to understand the persistence and transmission of intramammary infections. More recently, the focus has shifted to host-adaptation and pathogenesis studies, taking advantage of the availability of the whole genome sequence, challenge models and a variety of "-omics" approaches [108].

DNA-fingerprinting of *Strep. uberis* was first described around 1990 [71, 83, 208]. One early study suggested substantial strain homogeneity among *Strep. uberis* isolates [71], which may have been due to the fact that molecular typing methods were in their infancy and lacked discriminatory power. Since then, almost every study has shown a considerable level of heterogeneity among strains within and between herds, whether based on RAPD-typing [67, 90, 217], rep-PCR [207], PFGE [11, 46, 145, 203], or MLST [151, 193]. In one study, as many as 330 strains were detected among 343 isolates [46]. Despite the high level of heterogeneity within herds, an aseptically collected milk sample from an individual udder quarter usually contains a single strain of *Strep. uberis* [137, 145]. Even after experimental challenge of a mammary quarter with multiple strains of *Strep. uberis*, a single strain tends to become predominant [149]. Within a cow, multiple udder quarters may be infected with the same strain of *Strep. uberis*, which is usually interpreted as an indication of within-cow transmission [46, 98, 145, 207]. Persistent infection of a single quarter, resulting in repeated isolation of the same strain over time, is more common than simultaneous presence of a single strain in multiple quarters [118]. Infection of multiple cows within a herd with a single strain has also been described, and has been attributed to cow-to-cow transmission [46, 98, 145, 193]. In some herds, up to 50% to 100% of animals appear to be infected by the same or closely related strains of *Strep. uberis* based on RAPD-typing [67, 217] or PFGE [155]. *Strep. uberis* infections may be transient or they may persist over the non-lactating period, during lactation, or during short-term or extended antimicrobial treatment [125, 137, 145, 151, 207, 217]. It is also possible to find different strains before and after the non-lactating period [137] or when repeated episodes of clinical mastitis are observed in a single lactation [118].

As for *K. pneumoniae*, looking for mastitis-causing *Strep. uberis* in the environment is like looking for the proverbial "needle in a hay stack". Only 2 molecular

epidemiological studies of *Strep. uberis* in the dairy environment have been reported [110, 219]. Most environmental samples (87%) contain multiple *Strep. uberis* strains, with an average of 2.5 strains per sample based on ribotyping of 4 isolates per sample [219]. Heterogeneity of *Strep. uberis* is higher in soil samples than in fecal samples, and as many as 20 isolates per soil sample may need to be typed to find all strains that are present in the sample [45]. When comparing the strain distribution between environmental, fecal and milk isolates, many strains can be found in multiple sample types, and some strains are more common than others in environmental samples [110, 219]. These observations raise the possibility that the presence of a single strain in multiple quarters or cows in a herd may be due to exposure to a strain that is predominant in the environment, rather than to cow-to-cow transmission, which is the mechanism that is usually postulated.

The outcome of experimental challenge or natural infection with *Strep. uberis* may range from severe clinical disease to asymptomatic infection or even failure to establish infection [82, 193]. Several authors have tried to correlate strains with clinical or epidemiological characteristics such as persistence of infection, clinical signs or elevation of leukocyte counts in milk. Some studies support the existence of persistent strains [217] whereas others do not, leading to the suggestion that cow-factors rather than strains determine the duration of infection [151]. Alternatively, it is possible that none of the currently used molecular markers, which are largely based on primer binding sites, restriction sites, or sequences of housekeeping genes, are relevant indicators for duration of infection. Similarly, some studies support an association between strains and clinical signs or leukocyte counts [145, 193], whereas others do not [217]. The capsule gene *has*A has been associated with clinical mastitis in field studies [150] but challenge studies with *has*A deletion mutants demonstrated that the gene itself is not needed to cause clinical signs [55]. These results may potentially be explained by linkage of *hasA* to other virulence genes [150].

Despite the heterogeneity of the *Strep. uberis* population, 3 CCs have been associated with specific origins and manifestations [150, 193]. In the UK, CC5 is the most common CC found in milk, whereas CC143 is the most common CC in New Zealand. CC86 is found in Australia, New Zealand and the UK and is less common than CC5 or CC143 in all three countries. The prevalence of CC5 and CC86 did not differ between isolates from the environment, milk or body sites of cows in New Zealand. CC143 was overrepresented among environmental isolates, and isolates that have not been assigned to a CC were underrepresented in the environment compared to milk samples [150]. CC5 is predominantly associated with clinical mastitis, CC143 with subclinical mastitis and CC86 with latent infection, i.e. presence of *Strep. uberis* without a discernable inflammatory response [193]. These associations are not absolute, because clinical *Strep. uberis* mastitis is common among New Zealand dairy cattle despite the predominance of CC143, and subclinical *Strep. uberis* mastitis is common in the UK despite the predominance of CC5 [24, 118]. The 6-gene MLST scheme [218] has been used to identify STs that were unique to a single sample or common to multiple cows, herds or countries. Unique and common strains were compared with *Strep. uberis* O140J using a whole genome DNA microarray, with the aim to identify genetic elements that might explain why some strains are more common than others [104]. The microarray data showed that approximately 82.5% of the O140J genome can be considered core genome. Four genomic regions, which appeared to have arisen from LGT based on GC-content, were overrepresented among putatively host-adapted strains, but none of these elements were exclusive to one group of strains [104]. As for *Strep. agalactiae*, mobile genetic elements are suggested to play a role in the evolution and niche adaptation of *Strep. uberis*, but their role in *Strep. uberis* evolution seems limited compared to other Pyogenes group streptococci [35, 73, 162, 205]. A wide range of virulence genes may be present in *Strep. uberis* in a variety of combinations [158]. So far, no single set of virulence markers explains the different clinical and epidemiological manifestations of *Strep. uberis* mastitis. It seems reasonable to conclude that *Strep. uberis* is primarily an opportunistic environmental pathogen [205], and that it may show enhanced cow-to-cow transmission in some herds, possibly due to acquisition of MGEs that confer a survival or transmission advantage and possibly due to lapses in herd management.

Streptococcus dysgalactiae

The epidemiology of *Strep. dysgalactiae* subsp. *dysgalactiae* is poorly understood. It has been described as a contagious pathogen [60] and as an environmental pathogen [178], but environmental sources have not been investigated. Evidence for the dual nature of this pathogen comes from intervention studies conducted in the 1960s [132] and from molecular studies conducted in the 1990s. RAPD-typing was used to explore the persistence of *Strep. dysgalactiae* over the dry period [137]. Results from this study, which covered 12 quarters from 6 cows, illustrate a number of features of *Strep. dysgalactiae*. First, infections may be transient or persistent. Second, one strain seems to dominate within the herd. Finally, when multiple quarters are positive simultaneously, this is usually caused by a

single strain within a cow, hinting at within-cow transmission. In a nutshell, and with all the caveats appropriate for a single study with a limited number of cows, these patterns describe a mixture of what one would expect for typical contagious pathogens (persistent infections, dominant strain) and typical environmental pathogens (transient infections, multitude of strains). To date, all molecular epidemiological studies of *Strep. dysgalactiae* have yielded results that fit with a mixed contagious-environmental epidemiology. Baseggio and colleagues [11] conducted PFGE of 13 isolates from 8 herds in Australia and confirmed that shared and unique strains co-exist within herds. Gillespie and colleagues [67] used RAPD-typing to examine 116 isolates from 3 herds in the USA. Multiple strains were identified (n=17), but the majority of isolates (73%) belonged to one of two RAPD-types. One type was found in all 3 herds and was the most common type in two of them. The other type was found in 2 herds and predominated in one of them. Wang and coworkers [203] showed that in each of 3 herds they investigated, most or all of the infections were caused by the same strain, leading them to emphasize the likely importance of cow-to-cow transmission. The presence of the same predominant type in multiple herds [67, 203] raises the possibility of niche-adaptation of a specific subset of the *Strep. dysgalactiae* population, although alternative explanations such as contacts between farms could also be considered. Somewhat puzzling is Oliver's observation that cows tend to be positive for a single strain, which may appear in multiple quarters at quite distant time points (1998). Variable shedding of intramammary pathogens with a succession of culture-negative and culture-positive results has been described for *Staph. aureus* [172] but not for *Strep. dysgalactiae* and further investigation of this phenomenon may be warranted. By contrast, in an Australian study of 12 cows from 5 herds, persistence with continuous shedding seemed to be the standard [203]. After a gap of about 10 years, new studies on *Strep. dysgalactiae* mastitis are starting to appear. As discussed for *Strep. agalactiae* and *Strep. uberis*, mobile genetic elements may act as a vehicle for LGT between streptococcal strains and species, including transfer of virulence genes and antimicrobial resistance genes [73, 156]. The lactose-operon that is shared by *Strep. agalactiae* and *Strep. dysgalactiae* subsp. *dysgalactiae* could constitute a major survival advantage in the bovine mammary gland [162]. *Strep. dysgalactiae* subsp. *dysgalactiae* also shares genes with *Strep. pyogenes* and *Strept. equi* subsp. *zooepidemicus*, *Strep. uberis* and *Strep. suis* [156, 157, 185]. Population level studies to link clinical, epidemiological and genomic observations are lacking for *Strep. dysgalactiae* subsp. *dysgalactiae*. As stated by Wang and coworkers in [203] and still true today, "more work is required".

Other Streptococci, Enterococci and Lactococci

Other streptococci that are occasionally associated with bovine mastitis include *Strep. equi* subsp. *zooepidemicus* (SEZ; [50]) and *Strep. canis* [32, 75, 191]. Based on PFGE [75] or automated ribotyping [191], outbreaks of *Strep. canis* mastitis were due to a single strain of *Strep. canis* within a herd. In one case, circumstantial evidence was used to identify a cat with chronic sinusitis as the most likely source of infection [191]. Signs of infection in the cat predated signs of infection in the cows, and the cat had access to cows. Because interspecies transmission of *Strep. canis* is relatively rare, and because herd management was permissive to contagious transmission of mastitis pathogens, a single transmission event from cat to cow followed by cow-to-cow transmission was considered more likely than the cat acting as a point source for each individual cow [191]. This study exemplifies how molecular data need to be combined with other types of epidemiological data to infer transmission routes. Molecular epidemiological studies of bovine mastitis caused by SEZ have not been reported but in a recent SEZ-mastitis outbreak in goats, attempts were made to determine whether horses that were co-grazed with the goats could be identified as source of the outbreak [148]. As for the *Strep. canis* outbreaks, a single strain was associated with the SEZ outbreak. The mastitis-causing strain of SEZ, however, could not be identified in horses. It is possible that the goats contracted SEZ from elsewhere but failure to detect the outbreak strain in the horses was attributed to a delay in sampling of the horses relative to the mastitis outbreak, and to the heterogeneity of SEZ strains in equine feces [148].

In bovine mastitis diagnostics, streptococci are often grouped with other genera such as enterococci and lactococci. Because phenotyping is generally used for species identification, even though it is unreliable [87], limited information is available on the exact contribution of *Enterococcus* or *Lactococcus* species to mastitis. Strain typing studies of these organisms are almost no-existent. Petersson-Wolfe and colleagues [144] showed that enterococci from bovine mastitis were genetically diverse, in agreement with their probable origin from feces, as implied in the names of some of the most common species associated with mastitis: *E. faecalis* and *E. faecium*.

Staphylococcus Aureus

Staphylococcus aureus is a commensal and pathogen of humans and several animal species, including cattle. In women, *Staph. aureus* is among the most common etiological agents of bacterial mastitis but human staphylococcal mastitis has not been extensively studied, unlike

many other *Staph. aureus* induced conditions in humans [36]. By contrast, *Staph. aureus* is possibly the most studied mastitis pathogen in dairy cattle. Due in part to its importance as a human pathogen, many typing methods have been used or developed for *Staph. aureus* including, but not limited to, ribotyping, RAPD-typing, PFGE, MLST, spa-typing, coagulase gene RFLP (restriction fragment length polymorphism), MLVA, micro-arrays and whole genome comparisons [57, 80, 88, 178, 182]. More than for any other pathogen described in this paper, this section can only be a summary of the main findings from molecular epidemiological studies. Major themes include the mode of transmission, sources, strain-specific clinical or epidemiological manifestations and host association.

Based on epidemiological studies and mastitis control efforts, *Staph. aureus* has been classified as a contagious pathogen [60]. This classification is supported by molecular data, which show that in most herds with *Staph. aureus* mastitis, a single strain affects multiple cows and is often the most prevalent strain [7, 124, 188, 214]. Transmission is thought to occur primarily via the milking machine, udder cloths or milkers' hands. Molecular typing also supports a role of flies in transmission of *Staph. aureus* between animals [31, 68]. Successful control of *Staph. aureus* mastitis has been described [85, 178, 215], but prevention of cow-to-cow transmission often fails to eliminate the problem from dairy herds. Commonly used explanations for disappointing results from control efforts include false-negative results from bacteriological culture, resulting in undetected cases that may re-infect the rest of the herd, and the poor response of *Staph. aureus* mastitis to treatment [10]. Molecular studies offer an additional explanation. In most herds, numerous strains with low prevalence or incidence can be found in addition to one or a few high-prevalence strains [7, 31, 79, 106, 124, 188, 214]. Presence of multiple strains proves that not all infections are the result of cow-to-cow transmission and in some herds, *Staph. aureus* mastitis shows the molecular epidemiology and management response of an environmental pathogen [178, 215]. In exceptional cases, as many as 5 different strains have been isolated from milk of a single animal over time [178], a pattern that is thought to be unique to environmental pathogens. The number of strains per herd is higher on farms that purchase animals than in closed herds [79, 123]. Extramammary sources of *Staph. aureus* include cows' skin and body sites and the farm environment, e.g. bedding materials, insects, people, non-bovine animals, feedstuffs and air [31, 163]. One study showed that milking equipment may play a role in cow-to-cow transmission of strains from milk or teat skin, and that milk and teat skin contain distinct populations of *Staph. aureus* [216]. By contrast, Haveri and colleagues [79] found the same strains in milk, on teat skin, on milking equipment and milkers' hands. Only one strain showed a slightly higher prevalence among skin isolates than in milk samples. A third study [31] occupies an intermediate position, in that one predominant strain was shared between milk and body sites in most herds, in agreement with results from Haveri et al. [79], whereas a second strain was predominantly or exclusively found in milk or body sites only, in agreement with results from Zadoks et al. [216]. Similarity of strains from milk and skin was documented in studies on a small number of herds that were selected on the basis of *Staph. aureus* mastitis problems, whereas the difference between milk and skin strains was observed in a cross-sectional study of a large number of herds [31, 79, 216]. Strain distribution is herd-specific [31], so study design and herd selection may explain some of the discrepant results. Strains from milk and skin can both be found in the barn environment [31]. Among body sites, hock skin was specifically identified as a common site for colonization with milk- or skin-associated strains of *Staph. aureus* [31]. Some authors see extramammary presence of *Staph. aureus* as a potential source of intramammary infections but others favor the opinion that intramammary infection results in contamination of extramammary sites [31, 79].

Whereas *Strep. agalactiae* is generally easily controlled and *E. coli* populations are too heterogeneous to warrant investigation of strain-specific characteristics, the balance of strain predominance and heterogeneity in *Staph. aureus* is such that considerable effort has been invested in identification of strain specific outcomes of infection. This has led to demonstration of strain-specific associations with somatic cell count [39, 195, 214], milk yield [121], biofilm production [61], clinical signs [77, 214], persistence [78] and treatment response [39, 70, 195]. Not all studies support an association between clinical outcome and strain [106, 121, 124]. In some studies, the existence of an association depended on the typing method, treatment product or clinical parameter used [39, 78, 221]. Strain-specific differences in transmissibility have also been documented [70, 106, 122]. Some authors suggest that molecular markers of the likelihood of transmission or cure should be incorporated into diagnostic protocols [10, 70]. Apart from the *blaZ* gene for penicillin resistance, markers are currently not sufficiently standardized to be implemented routinely. Many staphylococcal enterotoxin genes can be present in bovine *Staph. aureus*, including staphylococcal enterotoxins (SE) A through D, G through O and U, toxic shock syndrome toxin (TSST) and exfoliative toxins A and B [78]. Presence of the combination of SEC/TSST is common in bovine *Staph. aureus* in many areas [58, 59, 131, 192] but virulence gene profiles are very heterogeneous and differ between regions and countries [78, 107, 202]. Staphylococcal enterotoxins may be relevant to human health because of their role in food

poisoning or clinical syndromes such as toxic-shock syndrome but they are not essential for the pathogenesis of bovine mastitis [106, 107].

Using comparative methods, predominance of strains within and across herds was recognized [57, 59, 106, 216]. It was also shown that, as for *Strep. agalactiae*, human and bovine *Staph. aureus* are largely distinct populations [106, 170]. Comparison across herds, countries, and host-species became much easier after the introduction of MLST [51]. This led to identification of STs and CCS that were found exclusively in animals, predominantly in humans, or across a wide range of host species [176, 182, 197]. Some STs can be found in cattle, goats and sheep, e.g. ST133 and ST126 [153, 176]. Other STs are limited to goats or cattle only, e.g. ST703 and ST151, respectively [176]. On-line databases can be skewed by underreporting if only type strains are entered in the database or by over-reporting of herd specific strains [175, 195]. Based on publications, the most common CC in bovine mastitis across herds and countries are associated with ST97, ST126, ST133, ST151, ST479 and ST771 [2, 76, 88, 153, 166, 174, 176, 182, 195]. The list of studies may appear long, but correlation of findings from different research groups would be facilitated if more groups included MLST in their typing protocols. Identification of host-associated lineages has led to the search for molecular correlates of host specialization [80]. Although lineage- and host-specific genes have been identified, many human and animal *Staph. aureus* strains are genetically quite similar [182]. Bovine strains are heterogeneous in content and no gene or open reading frame is uniformly shared by all bovine *Staph. aureus* strains [13, 101, 198]. LGT may contribute to the emergence of animal-pathogenic strains from human strains, and vice versa [72, 111]. Several STs have been associated with bovine mastitis as well as human colonization or infection, e.g. ST1, ST8, ST9, ST79 and methicillin susceptible or methicillin resistant ST398 [176, 195, 197]. In some instances, there is evidence of humans and cattle sharing the same strain of *Staph. aureus* through direct contact [95, 106, 170]. Of more concern is the emergence of supposed bovine-adapted strains such as ST130 or ST151 in the human population [65] and emergence of supposed human-adapted strains such as ST8 and ST20 as common strains in the cattle population [166]. This is particularly worrisome in the case of methicillin resistant *Staph. aureus* [65]. If human and bovine strains develop the ability to spread with equal ease in both host populations, zoonotic risks and the implications of antimicrobial resistance will become much greater than they are today.

Coagulase Negative Staphylococci

Coagulase negative staphylococci (CoNS) are a heterogeneous group of organisms with limited but non-negligible impact on udder health and productivity [171, 186]. For many years, CoNS were identified to the species level using phenotypic methods. Such methods are unreliable for bovine isolates, partly because most phenotypic assays were developed for applications in human medicine whilst some of the most common CoNS of cattle, such as *Staph. chromogenes*, very rarely occur in humans [168, 213]. To allow for study of the impact of individual CoNS species on mammary gland health, accurate species identification of large numbers of CoNS isolates is needed. The usefulness of numerous molecular methods for this purpose has been evaluated, including PCR-RFLP of the *gap*C gene [140], tRNA intergenic spacer PCR [184], amplified fragment

Table 1 Strain level molecular epidemiological studies of coagulase negative staphylococci from bovine milk and extra mammary sources

Species identification method	Strain typing method	Target species (number of isolates)	Epidemiological comparison	Reference
API staph system	PFGE	*S. chromogenes* (66) *S. epidermidis* (37) *S. hyicus* (38) *S. simulans* (10) *S. warneri* (7)	Within-herd: heterogeneity of CoNS populations	[69]
VITEK	PFGE	*S. chromogenes* (27) *S. warneri* (2) *S. xylosus* (5)	Within-cow: Persistence over dry cow period	[154]
API staph system	PFGE	*S. epidermidis* (36)	Within-herd: Clonality of strains with antimicrobial resistance	[169]
API staph, ribotyping	PFGE	*S. chromogenes* (46) *S. epidermidis* (4) *S. simulans* (21)	Within-herd: heterogeneity in milk, bovine body sites and humans	[187]
Conventional methods	PFGE	*S. epidermidis* (200)	Between host: Comparison of human and bovine strains	[190]

length polymorphism (AFLP) [146], (GTG)5-PCR typing [25] and sequencing of the housekeeping genes 16s rDNA [140], *tuf* [30], and *rpo*B [168]. The distribution of species in milk samples and the environment has been compared, which has led to a tentative classification of species as cow-associated (e.g. *Staph. chromogenes* and *Staph. epidermidis*), opportunistic (e.g. *Staph. haemolyticus* and *Staph. simulans*), and environmental (e.g. *Staph. equorum* and *Staph. xylosus*; [147]). Despite the flurry of molecular studies at the species level, strain typing studies to explore the environmental or contagious nature of CoNS mastitis in a manner similar to that described for *Staph. aureus* and Streptococci are rare. The molecular epidemiology of some of the most common CoNS species has been explored by means of PFGE (Table 1). Surprisingly, most of the bovine CoNS strain typing studies did not use molecular methods for species identification (Table 1).

Thorberg and colleagues [190] used 105 epidemiologically unrelated human and bovine isolates to establish that PFGE has excellent discriminatory power for typing of *Staph. epidermidis*. Subsequently, they investigated 2 herds in detail and demonstrated dominance of one or two types in each herd. The dominant types from milk were also isolated from skin of the people that milked the cows. Because isolation of *Staph. epidermidis* from human skin is more common than isolation from bovine skin, the authors conclude that humans are probably the main source of infection for cows [190]. The *Staph. epidermidis* population from bovine milk was much more heterogeneous in a study by Gillespie and coworkers [69], who identified 21 PFGE patterns among 37 isolates of 29 cows. Only 5 PFGE-types were shared by multiple cows in a herd, with a maximum of 5 cows per strain. The majority of strains were unique to a single cow. Antimicrobial resistance may contribute to clonal dissemination of *Staph. epidermidis* strains. Three of 5 multidrug resistant (MDR) strains were identified in multiple cows whereas only 2 of 17 non-MDR strains were identified in multiple cows [69, 169]. For *Staph. chromogenes*, considerable within-herd heterogeneity was observed based on AFLP and PFGE [69, 154, 187]. The heterogeneity of *Staph. chromogenes* is surprising because it suggests existence of environmental reservoirs. In one study, cows' body sites and milkers' hands were identified as a source of *Staph. chromogenes* [187], but other attempts to identify extra mammary reservoirs have been unsuccessful [147]. When multiple *Staph. chromogenes* isolates are obtained from a single quarter during lactation or before and after the dry period, this may be due to the same strain, suggesting persistence of infection, or to different strains, suggesting cure and re-infection [69, 154]. Similarly, *Staph. hyicus* infections may or may not persist over the dry period. In lactation, *Staph. hyicus* infection can last up to 10 months [69]. In theory, this could create a window of opportunity for contagious transmission but occurrence of the same strain in multiple cows or herds is rare [69]. Molecular data support the possibility that *Staph. simulans* could be cow-associated, and yet this species has also been classed as opportunistic [147, 187]. Data for other species are too sparse to allow for meaningful interpretation. Figure 3 illustrates the heterogeneity of staphylococcal species and strains that may be found within an animal. Given the level of within-herd and even within-cow heterogeneity of CoNS species and strains, strain typing will be essential for detailed studies of transmission, persistence and cure of CoNS infections in dairy cattle.

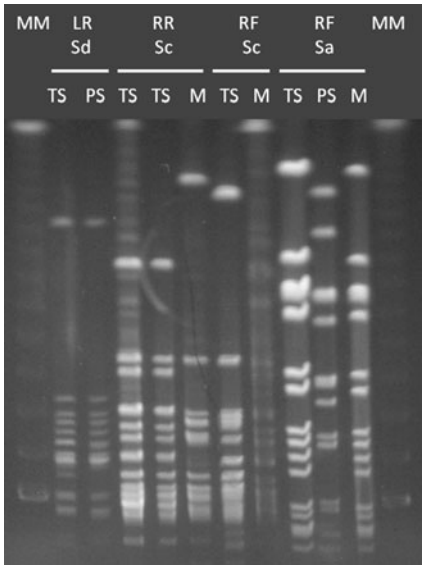

Figure 3 Pulsed field gel electrophoresis results for staphylococci from teat skin (TS), prepartum secretion (PS) and early lactation milk (M) of a single animal. Lanes 1 and 12 contain molecular markers (MM); Lane 2 and 3 represent *Staphylococcus devriesei* (Sd) from the left rear (LR) mammary quarter; Lanes 4 to 8 represent *Staph. chromogenes* (Sc) from the right rear (RR) and right front (RF) quarters; Lanes 9 to 11 represent *Staph. aureus* (Sa) from the right front quarter. One strain of *Staph. devriesei,* four strains of *Staph. chromognes* and two strains of *Staph. aureus* can be seen (Ringen and Middleton, unpublished data)

Other Mastitis Pathogens: *Prototheca* and *Mycoplasma*

Molecular methods for species-level identification have been developed for several other genera of mastitis pathogens, including *Prototheca*, a group of yeast-like micro-algae that have been described as a cause of mastitis in Japan [139], Europe [5, 89, 160] and North and South America [6, 28]. Several methods have been used for identification of species and subspecies genotypes of *Prototheca.* Genotype-specific PCR and RFLP and 18S rDNA sequence analysis are used to identify *P. zopfii* genotype 1 and genotype 2 and *P. blaschkae*, formerly

Table 2 Distribution of *Prototheca* isolates from milk and environmental samples over species and genotypes

Country	Milk			Environment			Reference
	P. zopfii 1	*P. zopfii* 2	*P. blaschkae*	*P. zopfii* 1	*P. zopfii* 2	*P. blaschkae*	
Japan	0	67	0	29	3	0	[139]
Germany	2	177	21	n.t.	n.t.	n.t.	[5]
Poland	0	43	1	n.t.	n.t	n.t.	[89]
Italy	0	105	3	0	45	8	[160]

n.t. not tested

known as *P. zopfii* genotype 3, [5, 89, 139]. Real-time PCR can identify *P. zopfii* genotype 2, *P. blaschkae* and *P. wickerhamii* [161]. When combined with DNA resolution melting analysis (qPCR/RMA), real-time PCR also allows for identification *P. zopfii* genotype 1, *P. stagnora* and *P. ulmea* [161]. *Prototheca zopfii* genotype 2 is the most common genotype in milk (Table 2). Some authors suggest that this indicates increased ability to cause mastitis because *P. zopfii* genotype 2 is rare in the environment compared to *P. zopfii* genotype 1 [139]. Other authors find *P. zopfii* genotype 2 as the most common genotype in milk as well as the environment [160]. Development of additional molecular methods, especially at the subspecies level, may aid in studies of the epidemiology of *Prototheca* mastitis.

Mycoplasma spp. are mollicutes, cell wall-less, slow growing organisms that require special culture media and growth conditions. For *Mycoplasma* spp., as for *Prototheca* spp., many molecular studies focus on detection and species identification, especially because molecular diagnostics are faster than culture [8, 81, 164, 165]. As for other bacterial mastitis pathogens, outbreak investigations and routes of transmission are the main subjects of molecular epidemiological studies. In contrast to most other mastitis pathogens, *Mycoplasma* spp. may affect multiple organ systems. Asymptomatic carriage in the ears and respiratory tract has been described, as well as otitis, pneumonia and arthritis, primarily in calves, and mastitis in prepubertal calves and adult cattle [61–63, 116]. The occurrence of various non-clinical and clinical manifestations adds new angles to *Mycoplasma* transmission studies because multiple age groups, carrier states and disease syndromes may act as source for mastitis outbreaks [152]. In addition, dissemination of the pathogen within the individual host, probably via hematogenous or lymphatic spread, may occur [61, 62]. The molecular epidemiology of respiratory *M. bovis* has been investigated with AFLP, PFGE and RAPD typing [116]. AFLP [102], PFGE [14] and restriction enzyme analysis (REA) [40] have been used to study the molecular epidemiology of mastitis-associated *Mycoplasma* spp., including *M. bovis, M. californicum* and *Mycoplasma* sp. bovine group 7. Within individual animals, multiple strains of the same *Mycoplasma* species can be found at different body sites or even within the mammary gland [14, 63, 102]. In most instances, however, *M. bovis* or *M. californicum* isolates found in milk, udder parenchyma and supramammary lymph nodes belong to the same PFGE type [14]. Furthermore, 90% of isolates from eyes, ears, feces, joints, the urogenital system and internal organs belong to the same PFGE type as the mammary strains [14], which could be explained by hematogenous spread. In the respiratory tract, only 40% of isolates belonged to PFGE types found in the mammary system and the rest of the body [14]. *Mycoplasma* is more heterogeneous in the respiratory tract and it is also found more commonly in the nose than other body sites [152]. Thus, it seems likely that colonization of the respiratory tract by a heterogeneous *Mycoplasma* population is occasionally followed by systemic dissemination of one or a few strains. *Mycoplasma* spp. may be transmitted vertically from dam to calf or horizontally via nasal discharge or from cow to cow at milking [40, 63]. Transmission and dissemination of *Mycoplasma* via extra mammary routes explains why control of *Mycoplasma* mastitis can fail when it is solely based on detection of intramammary infections and prevention of cow-to-cow transmission at milking [61, 62]. In Australia, *Mycoplasma* sp. bovine group 7, a member of the *M. mycoides* cluster, has been associated with mastitis. An REA-based study of 24 epidemiologically related strains and 36 epidemiologically unrelated strains from multiple herds, countries and continents showed that all epidemiologically related isolates belonged to the same strain, whereas 28 different strains were detected among 32 unrelated isolates [40]. REA did not have perfect typeability, but it did show good discriminatory power and excellent epidemiological concordance [180]. Without demonstration of discriminatory power, homogeneity of strains within an outbreak would not have been epidemiologically meaningful. As in the studies of *M. bovis*, multiple organs within an individual animal and multiple animals within a herd were infected by the same strain. This included calves, cows and aborted fetuses [40]. Both *M. bovis* and *Mycoplasma* sp. bovine group 7 may persist in a

herd for a long time, with documented persistence of a year and 18 months, respectively [40, 152]. Regional persistence of *Mycoplasma* strains for even longer periods may also occur. In Denmark, a single AFLP type was responsible for 2 cases of calf pneumonia and 2 multi-herd outbreaks of *M. bovis* mastitis. The cases of pneumoniae were detected at intervals of 10 years (1981 and 1991) in one region. The multi-herd outbreaks of mastitis occurred in 1984 and 1986/7 in two different regions [102]. The suggestion was made that the first pneumonia isolate gave rise to the first mastitis outbreak, which then led to the second mastitis outbreak. Contact between farms could have occurred via purchase of colonized or infected animals, attendance at animal shows or cross-contamination by service personnel visiting multiple farms [102]. The same report describes that *M. bovis* isolates obtained in the 1990s showed greater heterogeneity than those from the 1980s. Strains from the 1990s were primarily obtained from lung samples whereas strains from 1980 were largely obtained from mammary glands. It is unclear whether heterogeneity is associated with the different decades or the different organ systems. Considering that lung isolates were more heterogeneous than mastitis isolates, one could argue that the mastitis strain was particularly well suited to survival in the bovine mammary gland and that homogeneity indicated host adaptation of the strain rather than epidemiological connections between farms. As in many situations, this example illustrates that one has to be careful with interpretation of molecular data and that epidemiological data must be taken into consideration to identify and differentiate possible and likely scenarios [212]. The recent publication of the complete genome of *M. bovis* type strain PG45 [209] and the emergence of *Mycoplasma* mastitis in multiple countries [135] can be expected to lead to development of additional typing methods and to further studies of the epidemiology, pathogenesis and control of *Mycoplasma* mastitis. Given that MLST has already been developed for *M. agalactiae*, a mastitis pathogen of sheep and goats, and for *M. hyopneumoniae*, it seems only a matter of time until an MLST scheme for *M. bovis* is published [114, 117].

Summary and Outlook

Molecular epidemiological studies have not changed the biology of mastitis and despite decades of research, mastitis control in dairy herds still largely depends on conscientious and continuous effort on the part of the farmer or herd manager and his or her staff. Molecular epidemiological studies have, however, contributed considerably to our understanding of sources, transmission routes, and prognoses for many mastitis pathogens, which helps dairy farmers to focus their efforts in those areas most relevant to mastitis prevention and control. Molecular studies also contribute to an understanding of mechanisms of host-adaptation and disease causation, providing insight into pathogen evolution, potential targets for development of therapeutics or vaccines, and the risk of exchange of genetic elements or pathogens between bacterial species and host species. Molecular epidemiological studies will also be of value for understanding of mastitis in non-bovine host species, including sheep and goats. Many molecular epidemiological studies have been based on use of selected targets in the genome, giving rise to banding patterns based on restriction- or primer binding sites, or to allelic profiles for housekeeping or virulence genes. Such studies continue to be useful diagnostic tools when the aim is to understand pathogen sources and transmission mechanisms or to measure strain-specific clinical outcomes such as duration, severity and cure. The molecular epidemiology of mastitis has now moved into the genomics era, with whole genome sequencing completed for the major gram-positive pathogens and in progress for several gram-negative pathogens. Results from genomic studies and integration with other "-omics" such as host genomics, transcriptomics, proteomics and metabolomics, will give us yet more detailed understanding of the biology of mastitis. Years ago, Achtman [1] complained of "a surfeit of YATMs" (yet another typing method). The challenge for mastitis researchers is to avoid "a surfeit of YATSs" (yet another typing study) and to use molecular biology tools to generate a deeper understanding of mastitis epidemiology. The tools are available. Let's hope we manage to ask the right questions.

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